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13. ABSTRACT ***(Maximum 200 Words)***

Fibroblast growth factors consist of a family of ligands and tyrosine kinase receptors that are potent regulators of branching morphogenesis, organogenesis, and angiogenesis. We have constructed and characterized chimeric proteins that contain the intracellular kinase domain of FGFR1 linked to a modified FK506-binding protein. These chimeric proteins can be induced to homodimerize in the presence of the lipid soluble drug AP20187. The validity of this system was tested in the HC11 mammary epithelial cell line and in transgenic mouse models using a MMTV transgene cassette. In the mammary gland of transgenic mice treated with AP20187, a fifteen-fold increase in proliferation, activation of MAPK and AKT and a marked increase in lateral budding was observed when compared with wild-type littermates. Initial lateral buds appear after ten days of AP20187 treatment and consist of a single layer of epithelial cells retaining apical and basolateral polarity. After 2-4 weeks of AP20187 treatment, increasingly invasive lesions appeared with multi-cell layered lateral buds, loss of myoepithelium, increased vasculature branching and loss of cell polarity. These data suggest that short term FGFR1 kinase signaling can increase lateral budding of the mammary epithelium and sustained activation of this pathway results in alveolar hyperplasia and invasive lesions. We have developed an inducible system to study FGFR signaling in the mammary gland that will be useful in elucidating the role of these receptors in branching morphogenesis and provide a model system to understand early events in transformation

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Table of Contents

Introduction

Fibroblast growth factors (FGF) are potent growth factors involved in many developmental events during embryogenesis. FGFs play a role primary mesoderm induction, limb bud outgrowth and patterning, and lung alveogenesis. During these developmental events the expression of FGFs are restricted and regulated. The aberrant expression of FGFs outside these regulated confines has been correlated with tumorigenesis. The oncogenic roles of FGFs in mammary tumorigenesis were initially identified by the finding that mouse mammary tumor virus (MMTV) insertion can cooperatively activate Wnt-1 and FGF3 in tumors of MMTV-infected mice. Two other FGF family members have also been identified that cooperate with Wnt-1 to induce mammary gland tumorigenesis: FGF4 and FGF8.

We are interested in elucidating the function of aberrant expression of FGFs in mammary tumorigenesis. Overexpression of FGFs has been shown to induce mammary tumorigenesis in mouse models and has been postulated to play a role in human breast cancer. Dysregulation of FGFs and FGFRs has been reported in human prostate and breast cancers and is associated with poor prognosis. Understanding the mechanism of FGFs and FGF receptors (FGFR) in breast cancer may provide insight for new therapeutics derived to block FGF/receptor interactions or receptor tyrosine kinase activity. The goal of this project is to understand the mechanism of FGF8 and FGF receptors in tumorigenesis. To attain this goal we have developed a system for FGF-independent inducible activation of the FGFR to study the FGF signaling pathway *in vitro* and *in vivo.*

Body

The project outlined in this proposal sought to determine the oncogenic effects of Fgf signaling in the mammary gland. Previous studies in this laboratory have demonstrated that several Fgf family members are expressed during ductal morphogenesis in the mammary gland. The original project goal focused on the newly discovered, at that time, Fgf family member Fgf8. Fgf8 was demonstrated to be upregulated in several carcinoma cell lines and tumor specimens. The initial proposal focused on preliminary data generated in the lab that Fgf8 was upregulated in mouse tumor models while Fgf1 and Fgf2 mRNA levels were unchanged. In the normal mouse mammary gland Fgf8 was observed to increase during pregnancy, a period when other Fgf family members showed decreased expression levels. These data suggested that Fgf8 might have unique function in mammary gland development and tumorigenesis.

The initial Aims for this proposal were the following:

1) To determine the spatial and temporal expression of Fgf-8 and FGFRs during normal mammary gland development and in tumors.
2) To examine the expression and function of genes that may be regulated by Fgf-8, including Shh and Fgf-10, in the normal mammary gland and tumors.
3) To elucidate the function and tumorigenic potential of Fgf-8, Fgf-10 and Shh using an *in vitro/in vivo* model system.
4) To make targeted knockouts of Fgf-8 in the mammary gland using the cre/lox recombination system.

Technical Difficulties

Technical difficulties and the publication by another group of transgenic mice that express Fgf8 in the mammary gland changed the objectives of the proposed research. Modifications were made to the technical objectives and the Statement of Work a to address these issues. These changes were reported in the annual progress report for the 1998-99 year and were reviewed and approved by the USAMRC.

Aim #1. To determine the spatial and temporal expression of Fgf-8 and FGFRs during normal mammary gland development and in tumors.

The technical aspects of the original proposal relied on the availability immunological reagents directed against Fgf8. To study the expression and localization of Fgf8 protein in the mammary gland we raised eight antibodies against several different Fgf8 epitopes. Western blot experiments using some of these newly developed antibodies demonstrated affinity for recombinant Fgf8. Westerns from tissue extracts demonstrated that immunoreactive proteins migrated at expected size and were enriched by heparin-sepharose chromatography. In addition, these immunoreactive proteins were observed to be upregulated during pregnancy and in tumors and down regulated after ovariectomy. These observations were consistent with our previous results on mRNA expression for Fgf8. It was later determined, through the use of protein extracts from Fgf8 overexpressing and negative cell lines, that these immunoreactive proteins were cross-reactive proteins and did not represent bona fide Fgf8 isoforms. Further attempts to characterize Fgf8 protein expression using several antibodies from commercial sources and obtained from other laboratories were unsuccessful. Several biochemical techniques including heparin-Sepharose purification of protein extracts, modifications of the extraction process, immunoprecipitation, and affinity purification of antibodies have failed to produce quality Western blot or immunohistochemistry results.

Aim #3. To elucidate the function and tumorigenic potential of Fgf-8, Fgf-10 and Shh using an in vitro/in vivo model system.

Our initial proposal for this objective was to study Fgf8 transformation of the HC11 mammary epithelial cell line using an *in vitro/in vivo* model. We have made stable cell lines that express Wnts and FGFs and transplanted these cells and control non-transfected cells into the cleared fat pads of syngeneic host mice. These experiments have provided variable results due to the genetic instability of the HC11 cells possibly due to the presence of a mutated form of p53 in this cell line. Most transplants contained some degree of differentiated alveolar buds localized at the site of transplantation. There was, however, a high incidence of hyperplasia and abnormal growth surrounding all transplants including control experiments. Our conclusion from over thirty transplants is that the technique is highly variable and not useful for functional studies of Fgf8.

Aim #4. To make targeted knockouts of Fgf-8 in the mammary gland using the cre/lox recombination system

This original proposed objective was to make a targeted knockout of FGF8 in the mammary gland. The proposal was to develop mice that express the Cre recombinase in the mammary gland under the ß-casein promoter. These mice would then be bred with the floxed Fgf8 mice developed in Dr. Gail Martin's lab. The offspring from these mice would then be bred to initiate pregnancy inducing Cre expression in the mammary gland and causing recombination at the FGF8 lox sites. After further discussions with our collaborator (Dr. Gail Martin, UCSF) on this project we have determined that due to several potential difficulties the probability for success would be low. Since Fgf8 functions in a paracrine manner a very high recombination rate would be required. This is important to prevent the paracrine action of FGF8 secreted from unrecombined cells from compensating for the Fgf8 loss in knockout cells. Unfortunately, most of the existing transgenic lines expressing Cre recombinase in the mammary gland display patchy expression patterns do to the incomplete penetrance of the transgenes. Furthermore, other FGF family members expressed in the stroma and mammary epithelium may compensate for the loss of Fgf8 during development.

Modified Aims

During the course of this proposed research a paper was published that described data from the development and characterization of Fgf8 transgenic mice. These mice expressed Fgf8 in the mammary gland under the control of the MMTV promoter, and developed adenocarcinomas in the mammary gland with metastases to the lungs. Since our proposal had a similar objective to establish a functional mouse model to study Fgf8 oncogenic potential the technical objectives were modified to limit redundancy in this area. The modified objectives addressed the broader question of how Fgfs and Fgfrs interact to induce mammary gland tumorigenesis. The modified technical objectives were as follows:

1) To determine the spatial and temporal expression of Fgf-8 during normal mammary gland development and in tumors.
2) To examine the expression and function of genes that may be regulated by Fgf-8 and FGFRs in the normal mammary gland and tumors.
3) To characterize the cooperative potential of Fgf-8 and beta-catenin in mammary tumorigenesis.
4) Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

The modified Statement of Work, as approved by the USAMRC, is appended to this document for further review.

Modified Technical Objectives

Aim #1 To determine the spatial and temporal expression of Fgf-8 during normal mammary gland development and in tumors

Using the limited reagents available, the expression profile for Fgf8 was examined during mammary gland development and in tumors using RT-PCR and RPA analyses. These data demonstrated that several isoforms of Fgf8 were expressed during pregnancy and lactation but expression was lower in the mature and immature virgin mouse. No

expression of Fgf8 was detected in mammary glands from mice that were ovariectomized. In mammary gland tumors derived from tumorigenic cell lines, Fgf8 expression was detected at its highest levels. The spatial expression of Fgf8 in the normal mammary gland was localized to the epithelial component by tissue fractionation and RT-PCR analysis. These data demonstrated that Fgf8 is expressed during normal mammary gland development and may be hormonally regulated. Overexpression of Fgf8 was associated with tumorigenesis suggesting it may function in oncogenic transformation.

Aim #2 To examine the expression and function of genes that may be regulated by Fgf-8 and FGFRs in the normal mammary gland and tumors.

Transduction of MECs

The development of an alternative technique to transgenic mouse models to analyze gene function in the mammary gland would provide a significant advance in this field. The unique capacity of the mammary gland to regenerate upon transplantation makes this system adaptable to *in vitro* manipulation. The introduction of genes into MEC primary cultures prior to transplantation will allow mammary gland biologists additional means to study gene function without the cost, time, and variability associated with generating transgenic mice. This aim sought to develop an *in vivo* functional model system in which Fgfs can be analyzed for their developmental and oncogenic effects in the mammary gland. In addition, this technique would provide a tool for studying genes that may be regulated downstream of Fgf signaling pathways. The initial proposal was to utilize the mammary epithelial cell line, HC11, in a transplantable system. As indicated in "Technical Difficulties" for the original Aim #2 several problems arose with this system making the results uninterpretable. A new method was devised where MEC primary cultures were transduced using retroviral constructs containing Fgf8 and were then subsequently transplanted into syngeneic host mice.

Several retroviral vectors were designed for these experiments including constructs that contained Fgf8, ß-catenin, GFP and ß-galactosidase. It was determined using, the control ß-galactosidase retrovirus, that the transduction efficiency was very low for primary MECs. In addition to low infection rates in culture, the transplanted MECs did not produce X-gal-staining positive outgrowths. This led to several experiments that were designed to optimize the transduction and transplantation protocol.

Several methods were used to try to increase the MEC infection rates. This included culturing on different substrates, using all pseudo-type retroviruses and lentiviruses available, infecting in the presence of polybrene and retronectin, co-culturing MECs with packaging cells and spin infection. The optimization of the MEC primary culture infection rate resulted in an increase in transduction by 20-fold. However, the percentage of transduced MECs using the optimized protocol was only about ten percent at best. In addition, using the optimized protocol with the Fgf8, ß-catenin, GFP and ß-galactosidase retroviruses resulted in no detectable expression levels of these constructs in the resulting outgrowths in mice. However, a low level of ß-galactosidase activity was detected in transduced rat MEC outgrowths. The disparity between ß-galactosidase activity in rat

and mouse MECs transplants is unclear. These experiments suggest that retroviral infection of MEC primary cultures is inefficient and further optimization will be required to utilize this technique in the functional analysis of gene expression in the mammary gland.

We hypothesized that if mammary epithelial stems could be isolated from primary cultures and infected using the optimized protocol an increase in transduced gene expression in outgrowths may result. This led to collaboration with Dr. Margaret Goodell's and Timothy Graubert's lab to identify and isolate mammary epithelial stem cells. This collaboration led to the identification and enrichment of a cell population from the mammary gland that retains many characteristics associated with stem/progenitor cells. These data have been submitted for publication. Experiments to determine if an enriched population of mammary epithelial stem cells can be transduced and transplanted with increased efficiency are currently being undertaken. Data from the optimization of MEC primary culture infection and the ongoing experiments to transduce stem/progenitor cells will be combined for publication once these experiments are completed.

Identification of genes regulated by Fgf signaling

The Fgf family consists of genes for over 22 ligands and four receptors. The complexity of this family is not only represented by the number of genes, but by the number of different receptor isoforms that are generated. The number of gene products observed makes this family one of the most complex growth factor families identified. To study the temporal and spatial expression and function of each individual Fgf isoform in the mammary gland would be difficult. However, the complexity of the Fgf family is limited at the receptor kinase domains. Although there are numerous receptor isoforms in the Fgf family, each isoform has one of only four different kinase domains. The kinase domain of the receptor is the functional intracellular component that transduces signal from the extracellular environment to the nucleus. Fgfr mediated signal transduction is initiated through ligand interaction with the extracellular domain resulting in receptor dimerization. The close proximity of two Fgfr kinase domains induces a transphosphorylation event causing the phosphorylation of several tyrosine residues on the pair of receptors. The phosphorylated receptor is then capable of interacting with SH2 domain adapter and signaling factors that interact with the phosphorylated tyrosine residues. Fgf induced signaling is known to activate several key signaling pathways including MAPK and PLC-gamma.

To study the biological effects mediated by the Fgf family we employed an inducible Fgf-independent activation system of the Fgfr. The basis of this system is the ability of the FK-506 binding protein-12 (FKBP12) to interact with its naturally occurring chemical ligand FK506. The interaction of FKBP12 with FK506 is at a stochiometric relationship of 1-to-1; thus the tethering of two FK506 compounds results in a bivalent drug (FK1012) with the capacity to interact with two FKBP12 proteins. With this strategy, chimeric proteins that contain the Fgfr kinase domain fused to FKBP12 can be induced to dimerize in the presence of FK1012. A synthetic analog of FK1012 used in these studies is AP20187, which has modified moieties to reduce interactions with endogenous FKBPs and specifically interact with a variant of FKBP12 containing a F36V mutation (FKBPv).

All four Fgfr genes were cloned with the FKBPv domain to create chimeric Fgfr-FKBPv constructs. These chimeric proteins contain at the amino terminal Fgfr intracellular kinase domain followed by two FKBPv domains and hemmagluttin epitope tag. Fgfr-FKBPv proteins also have at their amino terminal the Src myristlation sequence for targeting to the intracellular membrane surface. Three of the four Fgfr genes were characterized extensively including Fgfr1, Fgfr2 and Fgfr3 (R1, R2, R3FKBPv, respectively). A construct containing only the FKBPv domain was used as a control. All expression, retroviral and transgene constructs contain the myristlation and epitope tag elements described above.

Fgf induced survival response in NIH3T3 cells

The Fgfr-FKBPv constructs were initially transduced into the NIH3T3 fibroblast cell line for functional characterization. NIH3T3 cells require serum factors for survival and proliferation and thus provide a system in which to study Fgfr signaling in these processes.

R1, R2, R3-FKBPv and FKPBv stably transfected NIH3T3 cells were plated onto duplicate plates, grown to sub-confluence and placed in serum free media containing 30nM AP20187 dissolved in ethanol or ethanol alone as a control. Treated cells were then observed for morphological changes over a 72 hour time course. R1, R2, and R3-FKBPv cells in the absence of AP20187 showed distinct morphological features associated with apoptosis including membrane blebbing, rounding and detachment from the plate. Similar results were obtained with the pBK-neo and FKBPv stable cells treated with and without AP20187. However, plates containing R1, R2, and R3-FKBPv cells treated with AP20187 did not display apoptotic morphological features. In a 72hour time course there was a two-fold greater number of viable cells with AP20187 treatment as determined by MTS assay. Similar results were obtained when NIH3T3 cells were treated with recombinant Fgf8 (rFgf8). These data suggest that AP20187 treatment of cells stably transfected with Fgfr-FKBPv constructs induces a survival response similar to that observed with Fgf ligands.

The activation status of caspase-3 was assayed to determine whether Fgfr-FKBPv dimerization could inhibit the apoptotic response induced by serum removal. NIH3T3 cells were transduced with constructs containing R1, R2, R3-FKBPv or FKBPv alone. Quadruplicate wells were treated with an increasing concentration of AP20187 in the absence of serum and the activation of caspase-3 was determined after 24 hours. Caspase-3 activation was high in wells treated in the absence or at low concentrations of AP20187. Detectable inhibition of caspase-3 was observed at approximately 500 picomolar concentration of AP20187.

Rnase protection assays (RPA) were utilized to determine if FGFR signaling can regulate the transcriptional regulation or stability of mRNAs associated with cell survival. Using a commercial multi-plex RPA system, the mRNA abundance of Bcl and caspase family members in transduced NIH3T3 cells treated under serum starvation and in the presence or absence of AP20187 was analyzed. No change in the relative abundance of caspase

family mRNAs was observed using this system. However, an increase in the anti-apoptotic Bcl-2 and Bcl-xl mRNAs was observed when cells were treated with AP20187.

These data suggest that FGFR signaling can inhibit apoptosis by at least two mechanisms: post-translational modification of anti-apoptotic signaling factors and upregulation of mRNA encoding survival factors. The mechanism of Bcl-2 and Bcl-xl regulation is unknown but may occur through transcriptional regulation (possibly through STAT or CREB signaling) or increased mRNA stability.

Aim #3 To characterize the cooperative potential of Fgf-8 and beta-catenin in mammary tumorigenesis.

As described above, this aim had several technical difficulties that required optimization of retroviral transduction techniques. Due to the difficulty with retroviral transduction of primary MEC and the requirement for two retroviral integration hits (FGF8 and stabilized ß -catenin) the probablility of success was low. Although no direct progress was made towards this aim, alternative methods have been developed to study the cooperativety between FGFs and the Wnt/ß-catenin signaling pathway. These experiments will use a bigenic mouse model and MEC primary culture transplantation. The two transgenic mouse models include a conditional FGFR dimerization system in the mammary gland, described in detail in the appended draft manuscript, and another Cre-recombinase inducible stabilized ß-catenin transgenic model. Briefly, once a bigenic line is bred, MEC primary cultures will be made, infected with adenovirus containing Cre-recombinase and transplanted into host mouse cleared mammary fat pads. The transplanted MEC will produce a stabilized ß-catenin (due to recombination and deletion of ß-catenin's N-terminal degradation domain) and conditional FGFR1 activation. Host mice can be treated with AP20187 to activate FGFR signaling, thus FGFR1 signaling can be analyzed in a stabilized ß-catenin background to functionally assess the cooperativety between these signaling pathways *in vivo.* Another graduate student in the laboratory is currently carrying out these studies.

Aim #4 Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis

The conditional FGFR constructs FGFR1-FKBPv and FGFR2-FKBPv were cloned into a transgene cassette under the control of the MMTV promoter to direct expression in the mammary gland. Transgenic mice were generated with these constructs and the results from these experiments are currently being prepared for submission as a manuscript to Nature Cell Biology.. A draft of the manuscript is appended to this report and describes the experiments and results in detail.

Key Research Accomplishments

1) Optimization of the isolation and increased transduction of primary MEC using Lac-z reporter retroviruses and lentiviruses.

2) The identification and isolation of mammary epithelial stem cells.

3) Development of a system to conditionally activate FGFR signaling pathways..

4) Development of a mouse model system to study FGFR signaling in ductal morphogeneis and tumorigenesis in the mammary gland.

Reportable Outcomes

Publications

Welm, B., Tepara, S., Venezia, T., Graubert, T., Rosen, J., Goodell, M. Isolation and Characterization of Functional Mouse Mammary Gland Stem Cells. (submitted for publication)

Welm, B., Freeman, K., Spencer, D., Rosen, J. Inducible FGFR activation results in increased lateral budding and alveolar hyperplasia in the mammary gland. (in preparation)

Welm, B., Freeman, K., Spencer, D., Rosen, J. Inducible ligand-independent activation of the FGFRs as a model system to study FGFR signaling. (in preparation)

Freeman, K.W., **Welm, B.**, Rosen, J., Greenberg, N., and Spencer, D.M. Modulating Prostate Tumor Malignancy with Conditional Fibroblast Growth Factor (FGF) Receptors. (in preparation)

Welm, B., Tepara, S., Rosen, J. Optimization of retroviral and lentiviral transduction of mammary gland primary cultures for transplantation and reconstitution. (in preparation)

Presentations

Welm, B., Freeman, K., Wang, J., Spencer, D., Rosen, J., (2001) *Selected short presentation.* Inducible ligand-independent activation of the fibroblast growth factor receptors as a method to study mammary gland tumorigenesis. Gordon Research Conference, Mammary Gland Biology,

Abstracts

Welm, B., Freeman, K., Wang, J., Spencer, D., Rosen, J., (2000) Inducible ligand-independent activation of the fibroblast growth factor receptors as a method to study mammary gland tumorigenesis. Era of Hope, Department of Defense Breast Cancer Research Program Meeting.

Freeman, K.W., **Welm, B.**, Rosen, J., Greenberg, N., and Spencer, D.M. (2000) Modulating Prostate Tumor Malignancy with Conditional Fibroblast Growth Factor (FGF) Receptors. Keystone Symposia Signaling 2000.

Welm, B., Freeman, K., Wang, J., Spencer, D., Rosen, J., (2000) Inducible ligand-independent activation of the fibroblast growth factor receptors as a method to study mammary gland tumorigenesis. Keystone Symposia Signaling 2000.

Weston Porter, **B., Welm.**, K., He, O., Volpert, N., Bouck, and M., Zhang, Maspin functions as an angiogenesis inhibitor by inducing apoptosis of endothelial cells. Keystone Symposia, Metastasis and Invasion (2000)

Monique Rijnkels, **Bryan Welm** and Jeffrey M Rosen. (1998) Tissue-Specific Deletions in the Mammary Gland Using Cre/loxP. MD Anderson Cancer Symposium.

Welm, B, Kittrell, F., Medina, D., Rosen, J., Krnacik, S. (1996). Expression of Fgf-8 during murine mammary gland development and tumorigenesis. Mol. Bio. Cell. 7: 186a.

Conclusions

The aims for this fellowship were to elucidate mechanisms of FGF signaling in the mammary gland to understand their function during mammary gland development and association with tumorigenesis. To this end, we have developed a system in which FGFRs can be induced to dimerize, permitting analysis of FGF signaling function in conditional *in vitro* and *in vivo* models. The signaling function of three FGFRs was characterized in HC11 and NIH3T3 cells and two FGFRs were analyzed in transgenic mice. The results of work related directly to FGF signaling will be published in at least two peer-reviewed papers. An early draft of one of these papers is appended to this report and presents the details and conclusions about FGFR signaling in mammary gland transformation.

The accomplishments from this fellowship were completed in the face of several technical difficulties associated with the original proposal. Although these difficulties required divergence from the proposed research design, the end results were closely related to the focus of the original proposal to study FGF signaling in the mammary gland. Additionally, these technical constraints led to experiments designed to optimize mammary gland reconstitution resulting in the identification and purification of mammary gland stem/progenitor cells. The ability to purify stem/progenitor cells from MEC primary cultures will potentially increase the efficiency of MEC transduction and reconstitution allowing for functional analysis of genes in the mammary gland without requiring transgenics. Additionally, as potential targets of carcinogen and oncogenic transformation the identity of mammary gland stem cells has implications in breast cancer research.

Future studies will be carried out using the conditional FGFR mouse model to elucidate mechanisms of early transformation events in the mammary gland. Transforming events

including conversion of mammary epithelium from single to multi-cellular layers, loss of cell polarity, angiogenesis, and metastasis can be induced in progressive steps and observed histologically and molecularly using this system. Other ongoing projects include breeding FGFR transgenic mice with other mouse models to understand the relationship of FGFR signaling with cooperative oncogenic pathways. Finally, studies are ongoing for the optimization of retroviral transduction of MEC primary cultures and analysis of mammary epithelial stem cells.

Appendix

Statement of Work
(updated 5/23/99)
Fgf-8 Expression and Function during Murine Mammary Gland Development and Tumorigenesis

Task 1 : To determine the temporal and spatial expression of Fgf-8 and FGFRs during mammary gland development and in tumors. (months 1-12)

a. Finish preliminary experiments on Fgf-8 expression during mammary gland development and in tumors. (months 1-8)

b. Examine the expression of FGFRs during normal mammary gland development. (months 1-8)

c. Characterize expression of FGFRs in mammary gland tumors.(months 1-10)

Task 2 : Examine the expression and function of genes that may be regulated by Fgf-8 and FGFRs in the normal mammary gland and tumors (months 4-15)

a. Determine the temporal and spatial expression of Shh and Fgf-10 during mammary gland development. (months 4-15)

b. Examine the expression of Shh and Fgf-10 in mammary gland tumors. (months 4-15)

c. Develop system of inducible activation of the FGFR. (months 8-14)

d. Identify and characterize genes that are regulated by FGFR activation in mammary epithelial cells. (months 14-30)

Task 3 : Characterize Fgf-8 tumorigenicity in the mammary gland. (months 1-24)

a. Clone Fgf-8 isoforms into retrovirus vectors. (months 5-10)

b. Optimize primary culture conditions and retrovirus infection efficiency. (months 8-13).

c. Transplant Fgf-8 infected primary cultures into syngeneic mice. (months 12-24)

d. Prepare and characterize outgrowths. (months 16-24)

Task 4: Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

a. Construct FGFR1-FKBPv and FGFR2-FKBPv transgenes. (months 12-14)

b. Inject transgenes. (months 14-15)

c. Characterize transgenic mice. (months 16-36)

Unpublished

Inducible FGF-independent activation of the FGFR in the mammary gland induces alveolar hyperplasia

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Abstract

Fibroblast growth factors consist of a family of ligands and tyrosine kinase receptors that are potent regulators of branching morphogenesis, organogenesis, and angiogenesis. We have constructed and characterized chimeric proteins that contain the intracellular kinase domain of FGFR1 linked to a modified FK506-binding protein. These chimeric proteins can be induced to homodimerize in the presence of the lipid soluble drug AP20187. The validity of this system was tested in the HC11 mammary epithelial cell line and in transgenic mouse models using a MMTV transgene cassette. In the mammary gland of transgenic mice treated with AP20187, a fifteen-fold increase in proliferation, activation of MAPK and AKT and a marked increase in lateral budding was observed when compared with wild-type littermates. Initial lateral buds appear after ten days of AP20187 treatment and consist of a single layer of epithelial cells retaining apical and basolateral polarity. After 2-4 weeks of AP20187 treatment, increasingly invasive lesions appeared with multi-cell layered lateral buds, loss of myoepithelium, increased vasculature branching and loss of cell polarity. These data suggest that short term FGFR1 kinase signaling can increase lateral budding of the mammary epithelium and sustained activation of this pathway results in alveolar hyperplasia and invasive lesions. We have developed an inducible system to study FGFR signaling in the mammary gland that will be useful in elucidating the role of these receptors in branching morphogenesis and provide a model system to understand early events in transformation

Introduction

The fibroblast growth factor family consists of over twenty ligands and four receptor tyrosine kinase genes that have been shown to be important regulators of

angiogenesis and embryionic organogenesis. Loss of function experiments have demonstrated that Fgfs are critical factors for limb bud outgrowth, lung development, gastrulation and brain development. In the adult, aberrant expression and misregulation of FGF ligands and their receptors is associated with breast and prostate tumorigenesis. Understanding the *in vivo* function of FGFs and FGFRs will be important for elucidating mechanisms of cellular transformation.

FGFR consists of a ligand binding extracellular region made up of several IgG loop domains that confers ligand affinity. FGF ligand and heparin sulfate interact with the FGFR in a tripartite complex to induce dimerization of the receptor. Dimerization of FGFRs results in activation of the intrinsic tyrosine kinase and trans-phosphorylation of the intracellular domain leading to SH2 protein interacting sites. FGFRs can activate several signaling factors including Ras, PI3 kinase, and PLC-gamma, thus, providing signals for proliferation, differentiation, and survival.

The expression of FGF ligands and receptors has been observed to be developmentally regulated in the mammary gland. The developmental expression profile for FGFs in the mammary gland suggests that they are generally expressed during ductal morphogenesis and decrease in pregnancy and lactation. Ductal morphogenesis is a highly proliferative stage during mammary gland development where the gland invades the stromal fat pad and establishes a ductal network. Additionally, expression of a dominant negative FGFR2 in the mammary gland during pregnancy results in inhibited lobuloalveolar development. This developmental expression profile suggests that FGF signaling may contribute to normal mammary gland development.

The initial observation that aberrant expression of FGFs can lead to mammary gland tumorigenesis was that MMTV insertional-activation of FGF transcription can induce mammary gland tumors. In the MMTV insertional assay, the ligands FGF-3, FGF-4 and FGF-8 were found to promote tumorigenesis. The direct transforming capacity of FGFs in the mammary gland was later confirmed using transgenic mouse models. These models, however, utilized overxpression of FGF ligands, thus, the compartmental effects mediated by FGF stimulation of epithelium and stroma during transformation is difficult to asses. Additionally, dysregulation of FGFs and their receptors has been observed in breast cancer cell lines and in about 20% of primary tumor samples. These data suggest that FGF signaling may have potent transforming capacity in the mammary gland.

The expression of FGFR1 during murine mammary gland development and its observed dysregulation in breast cancer have suggested a potential role for this factor in the growth, differentiation and transformation of the mammary gland. In this study, *in vivo* action of FGFR1 signaling, specically and conditionally in the mammary epithelium, is described during the early events in mammary gland tumorigenesis. To elucidate the function of FGFR1 signaling in the mammary gland an inducible FGFR-dimerization/activation system was developed and characterized in NIH3T3 fibrloblasts, mammary epithelial cell lines and *in vivo* using transgenic mice. Activation of FGFR signaling in mammary epithelium resulted in progressively invasive lesions occurring within a 2-4 week window of dimerizer treatment. FGFR1 kinase induced lesions were characterized for cell polarity, hormonal dependence, invasive properties, and

reversibility. This paper describes an inducible model for mammary gland tumorigenesis and is the first demonstration of an inducible dimerization system in transgenic mice.

Results

Inducible FGFR1-FKBPv construct

To study the biological effects mediated by the FGF signaling we employed an inducible FGF-independent activation system of the FGFR. The basis of this system is the ability of the FK-506 binding protein-12 (FKBP12) to interact with its naturally occurring ligand FK506. The interaction of FKBP12 with FK506 is at a stochiometric relationship of 1-to-1; thus the tethering of two FK506 compounds results in a bivalent drug (FK1012) with the capacity to interact with two FKBP12 proteins. This system has been used to study the dimerization induced signaling of several proteins in including Erb2, T-cell receptor, and caspases. With this strategy, chimeric proteins that contain the FGFR kinase domain fused to FKBP12 can be induced to dimerize in the presence of FK1012. A synthetic analog of FK1012 used in these studies is AP20187 which has modified moieties to reduce interactions with endogenous FKBPs and specifically interact with a variant of FKBP12 containing a F36V mutation (FKBPv). The FGFR1-FKBPv fusion proteins contain an N-terminal myristlation sequence, intracellular FGFR1 kinase domain, two FKBPv domains and C-terminal hemmagluttin (HA) epitope sequence (Fig. 1a). All four FGFR genes were cloned into this system, however FGFR1 will be described in detail here. A control FKBPv construct was designed that contains all domains except the FGFR sequence.

AP20187 dependent FGFR1-FKBPv signaling *in vitro*.

Dimerization of the FGFR results in autophosphorylation of tyrosine residues in the receptor. The phosporylation sites on the receptor provide docking sites for adapter and enzymatic proteins resulting in their phosphorylation. The FGF induced signaling cascade is known to activate several signaling factors including Grb2, Shc, Frs2, Plc-gamma, and the Ras/Raf/MAPKs. To examine the capacity of the dimerized FGFR1-FKBPv proteins in activating signaling pathways , the phosphorylation levels of FGFR1-FKBPv, MAPK and AKT were determined in the NIH3T3 and HC11 cells. An increase in tyrosine phosphorylated FGFR1-FKBPv was deteceted after AP20187 treatment of FGFR1-FKBPv transduced NIH3T3 cells by immunoprecipitation using either phospho-tyrosine or HA epitope antibodies (fig. 1h and data not shown). Phosphorylation of the signaling factors MAPK and Akt was also detected in AP20187 treated NIH3T3 and HC11 cells (fig.1I). FGFR1-FKBPv induced activation of MAPK and Akt were detected within 5 minutes

The FGFR1-FKBPv and FKBPv constructs were functionally tested in mammary and fibroblast cell lines to determine if they could recapitulate FGF signaling in an AP20187 dependent system. A characteristic of NIH3T3 fibroblast cells is that they require serum factors for survival and proliferation. This serum requirement makes them useful in assays to determine the proliferative or cell survival function of proteins. These cells were used to functionally analyze the FGFR-FKBPv constructs in regards to proliferations, apoptosis and downstream signaling factors.

FGFR1-FKBPv and FKPBv stably transfected NIH3T3 cells were plated onto duplicate plates, grown to sub-confluence and placed in serum free media containing

30nM AP20187 in ethanol or ethanol alone. Treated cells were then observed for morphological changes over a 72 hour timecourse. FGFR1-FKBPv cells in serum-free media and the absence of AP20187 displayed distinct morphological features associated with apoptosis including membrane blebbing, rounding and detachment from the plate surface. Similar results were obtained with the pBK-neo and FKBPv stable cells in serum-free media and treated with and without AP20187. However, plates containing FGFR1-FKBPv cells treated with AP20187 remained attached to the plate, formed distinct multicellular foci and did not display apoptotic morphological features. In a 24 hour timecourse there was a twofold greater number of viable cells with AP20187 treatment as determined by MTS assay (data not shown). The morphological and quantitative analysis was similar to that observed with recombinant FGF8 treated NIH3T3 cells (data not shown). These data suggest that AP20187 treatment of cells stably transfected with FGFR1-FKBPv constructs induced a survival response and blocked contact inhibition in NIH3T3 cells.

In response to serum removal, NIH3T3 cells undergo programmed cell death mediated through the caspase cascade. To quantitate the FGFR1-FKBPv induced inhibition of apoptosis, caspase-3 activation was assayed in serum starved FGFR1-FKBPv transduced NIH3T3 cells in the presence or absence of AP20187. Serum containing media was removed from the cells and serum-free media was added with or without AP20187 for 24 hours. The concentration of AP20187 was titrated to determine a dose response for caspase-3 inhibition. A significant decrease in caspase-3 activity was detected with about 0.5nM AP20187. A maximal decrease was achieved with 30nM AP20187. No significant decrease in caspase-3 activity was detected with the control

dimerization construct FKBPv. An AP20187 dependent increase in mRNA for the anti-apoptotic factor Bcl-xl was also observed (data not shown).

FGFR signaling has also been implicated in inducing a proliferative response in several cell types. To determine if the FGFR signaling pathway can induce proliferation in different cell types, cell cycle analysis was employed in AP20187 treated FGFR1-FKBPv transduced cell lines. HC11 mammary epithelial cell lines can be induced to produce milk proteins with lactogenic hormones, have limited alveolar outgrowth potential and are not tumorigenic. Transduced NIH3T3 fibroblast and HC11 mammary epithelial cell lines were treated with AP20187 in serum free media and their cellular DNA content was measured by fluorescence activated cell sorting (FACS). FGFR1-FKBPv NIH3T3 cells showed no change in proliferation with or without AP20187. However, FGFR1-FKBPv HC11 cells showed greater than a two-fold increase in proliferation in response to AP20187. In the presence or absence of AP20187 no difference in proliferation was detected in the control FKBPv HC11 or NIH3T3 cells.. These data suggest that FGFR1 regulated signaling pathways exert functionally different effects that are cell type dependent. Although FGFR1 can induce activation of the same downstream factors in fibroblasts and mammary epithelial cells, the proliferative effect mediated is dissimilar and may be a result of differing signal duration or signaling components between cell types

FGFR1-FKBPv Transgene

FGFR1 is expressed during the highly proliferative and morphogenic stage of mammary gland ductal morphogenesis. During ductal morphogenesis the mammary

gland establishes a ductal network through allometric proliferation and branching morphogenesis and is a period when the mammary gland is most susceptible to caricinogenic and oncogenic insults. Additionally, dysregulation and overexpression of FGF ligands and receptors has been observed in breast cancer biopsies and cell lines. To assess the *in vivo* function of FGFR signaling in the mammary gland, transgenc mice were developed that express the construct under the control of the mouse mammary tumor virus (MMTV) promoter. The MMTV promoter has been used in several transgenic mouse models to analyze gene expression in mammary gland epithelium during most stages of post-natal development including ductal morphogenesis, pregnancy and lactation. Targeted expression of the inducible FGFR1 construct using MMTV would allow for analysis of direct effects of FGFR1 kinase function in the mammary epithlelium during post-natal mammary gland development.

FGFR1-FKBPv and FGFR2-FKBPv constructs were cloned into an MMTV transgene cassette for the generation of transgenic mice. Three FGFR1-FKBPv and four FGFR2-FKBPv lines were generated that had germline transmission. Of these seven lines, three (1 FGFR1-FKBPv and 2 FGFR2-FKBPv) expressed the transgene in the mammary gland (fig.2m and data not shown). All three expressing lines displayed normal virgin morphology and similar inducible phenotypes described below. Further characterization was conducted on the FGFR1-FKBPv line 4775 which expressed low levels of mRNA, detectable by RT-PCR, and the lowest level of protein expression as determined by Western blot analysis.

Activation of FGFR1 results in increased lateral buds

To analyze gross morphology of mammary glands in the expressing transgenic lines, wholemount analysis of the thoriac and inguinal glands was performed. Six week old transgenic and control mice were treated with AP20187 by intraperitoneal injection every three days for 16 days and inguinal mammary glands were biopsied for whole mount and immunofluorecesnce analysis (Fig1...). Whole mount analysis of the mammary epithelium showed no gross morphological differences between age-matched non-transgenic liitermates treated with AP20187 and transgenic littermates treated with diluent (Fig2...) However, transgenic mice treated with AP20187 showed distinct lateral budding along the ductal epithelium. AP20187 induced lateral budding was observed throughout the mammary gland and appeared on primary, secondary and tertiary ductal branches. Increased lateral budding was observed at the distal tips of the mammary epithelium. Histological analysis revealed extensive alveolar buds, with single and multi-layered epithelium, lining the ducts. The alveolar buds did contain lumens and many lumens appeared constricted or convoluted. A similar phenotype was observed in all three FGFR-FKBPv expressing lines including one FGFR1-FKBPv (line founder number 4775) and two FGFR2-FKBPv (line founder numbers 4807 and 4808) lines treated under the same conditions. Due to the similarity of the phenotype, further analysis was performed in the FGFR1-FKBPv (4775) line.

AP20187 induced lateral buds do not require ovarian hormones

To determine if ovarian hormonal regulation is required for the lateral budding phenotype, transgenic mice were ovariectomized (ovex) and treated with AP20187. FGFR1-FKBPv transgenic mice were ovariectomzed and two days later injected with

AP20187 i.p. every three days for sixteen days. Whole mount analysis of ovex non-transgenic littermates treated with AP20187 showed constriction of ductal epithelium and a loss of terminal end buds (TEB) consistent with the loss of inductive effects from estrogen and progesterone. However, when ovex transgenic mice were treated with AP20187, dilated ductal epithelium and large bloated structures at the distal tips of the ductal network were observed. The ductal network did not completely fill in the fat pad in either transgenic or non-transgenic ovex mice treated with AP20187 suggesting that FGFR1 activation cannot rescue normal ductal elongation in the ovex background. However, the observation of FGFR1 induced lateral buds in the ovex background suggest that ovarian hormones are not required for this phenotype.

Transgene expression is localized to ductal and lateral bud epithelium

The localization of transgene expression in the mammary gland of transgenic mice was determined by immunofluorescence against the HA-epitope of the FGFR1-FKBPv protein. Mammary epithelial cells expressing the HA-epitope were observed in transgenic mice but not in non-transgenic littermates (Fig1...). Expression of the FGFR1-FKBPv protein was localized to the periphery of the cell and no nuclear staining was apparent, consistent with membrane targeting by the myristlation sequence. Transgene protein was punctate along the ductal epithelium of untreated transgenic mice (data not shown) while AP20187 induced lateral buds were strongly immunoreactive. It is possible that the abundant immunofluorescence of HA-epitope within lateral buds results from clonal expansion of transgene positive cells or auto-regulation of the MMTV promoter by FGFR1 activation. Lateral buds contained a mixture of mutually exclusive cell types either transgene positive or progesterone receptor (PR) positive (data not

shown). PR positive cells within lateral buds and lining ducts were BrdU negative consistent with the observed quiescent nature of PR expressing cells in the mammary gland. These data suggest that lateral buds comprise of multiple mammary epithelial lineages including mutually exclusive cell types expressing transgene or PR. If lateral buds result from expansion of FGFR1-FKBPv expressing cells these observations would suggest that transgene expressing cells may still retain the ability to differentiaite into PR expressing cells.

Progressive changes in mammary epithelial morphology

Whole mount, histological, and immunofluorescent analysis was utilized to characterize the progressive nature of the FGFR1-FKBPv induced lesions. To morphologically characterize lesions, whole mounts and paraffin embedded sections were stained with hematoxylin or hematoxylin and eosin (H&E), respectively. In addition, cell polarity was characterized using immunofluorescence against specific tight junction (ZO-1) and basement membrane (laminin) markers. FGFR1-FKBPv induced lesions were classified into three morphologically distinct types. Type I lateral buds were identified by the presence of a single layer of cubodial epithelium surrounded by myoepithelial cells, and with basement membrane localized laminin and apical (tight junction) localized ZO-1. Type-I lesions appeared within ten days of AP20187 treatment and were morphologically similar to lateral alveolar buds induced in early pregnancy. However, unlike lobuloalveolar development appearing in late pregnancy, the FGFR induced lateral buds remained closely associated with their primary ducts and did not completely occupy the interductal stroma.

After about two weeks of drug treatment, multi-bud type-II lesions appeared along the primary duct and were the predominant morphology of the mammary gland in AP20187 treated transgenic mice. Type II lesions contained multiple cell layers, had collapsed lumens (as determined by ZO-1 immunofluorescence), and expressed progesterone receptor. Laminin immunofluorescence of type II lesions revealed that cells at the periphery of the buds still remained organized around the basement membrane. In addition, these lesions remained closely associated with primary ducts and were not highly invasive into the surrounding stromal.

Type III lesions first appear after about four weeks of AP20187 treatment and had marked loss of laminin and ZO-1 expression. Type III lesions are multi-cellular, invasive, and highly vasculaized. Type III lesions were often associated with a surrounding leukocyte infiltration and reactive stroma.

FGFR1-FKBPv dimerization induces proliferation and activation of signaling factors

FGF-dependent dimerization of FGFRs induces transphosphorylation and activation of the intrinsic receptor kinase. Receptor kinase activation leads to phosphorylation of signaling factors eventually resulting in alteration of gene expression, proliferation and differentiaition. Dimerization induced phosphorylation of the FGFR1-FKBPv protein and signaling factors was analyzed in transgenic mice 24 hours after i.p. injection of AP20187 by Western blot analysis. Protein extracts isolated from AP20187 treated and untreated transgenic littermates showed increased FGFR1-FKBPv, AKT and MAPK phosphorylation in drug treated mice. In transgenic mice treated for 16 days, localization of the phosphorylated and active MAPK correlated with the phenotypic

lateral bud lesions as observed by immunofluorescence. These results suggest that AP20187 treatment can induce phosphorylation and activation of the FGFR signaling cascade *in vivo*.

To asses whether AP20187 induced lateral buds are hyperproliferative in response to activated FGFR signaling, BrdU uptake was quantitated in lateral buds. To assay proliferation, BrdU was administered to AP20187 treated and untreated transgenic mice 2 hours prior to sacrificing. Immunofluorsecence against BrdU was performed to localize BrdU uptaking cells in the mammary epithelium. In untreated transgenic mice, about 1% of mammary epithelial cells stained positive for BrdU. However, after 16 days of AP20187 treatment BrdU label was found in about 10% of mammary epithelial cells. The number of BrdU label found in ductal and lateral buds ranged from about 9% in lateral buds to 12% in ducts. These results suggest that activation of the FGFR kinase can induce proliferation not only in regions of phenotypic alveolar buds but also in ductal epithelium.

FGFR1-FKBPv activation alters mammary epithelial cell polarity and anokisis

Mammary epithelial cells demonstrate an intrinsic apoptotic mechanism upon detachment from their basement membrane substratum, a process termed anoikis. Epithelial cell interactions with the basement membrane proteins induces an intergrin mediated signal required for survival. Confocal microscopy was utilized to determine if the FGFR1-FKBPv induced lesions contain multi-cellular layers detached from the basement membrane. Thick frozen sections of mammary glands from mid-pregnant and AP20187 treated transgenic mice were stained with E-cadherin and laminin antibodies.

Immunofluorescent analysis demonstrated that mammary epithelial cells form a single cell layer contacting the laminin rich basement membrane and lumen in wild-type mid-pregnant mice. Additionally, E-cadherin localization was observed only on lateral sides of epithelial cells, at sites of cell-to-cell contact, and not on apical or basal membranes. Transgenic mice treated with AP20187 formed multi-cellular layers with inner (luminal) layers detached from the basement membrane. In drug-treated transgenic mice, E-cadherin staining was observed on all sides of epithelial cells suggesting cell-to-cell contact is no longer limited to lateral membranes. The loss of attachment to the basement membrane, peripheral localization of E-cadherin and mutli-cellular layering of the epithelium suggests that FGFR1-FKBPv induced lesions lose cell polarity and the ability to undergo anoikis.

FGFR1-FKBPv induced lesions have invasive characteristics

The mammary gland consists of multiple cell types including stromal, myorepithelial and luminal epithtelial cells. Between the luminal epithelial and the stromal cells lies a cellular and proteinaceous barrier composed of myoepithelial cells and extracelluar matrix.(ECM) proteins. During mammary gland tumorigenesis, epithelial cells acquire invasive characteristics including the capacity for regulation and remodeling of the luminal-to-stroma barrier. The upregulation of ECM degrading proteases and the loss of myoepithelial cells has been correlated with highly-invasive poor-prognostic tumors in humans. In addition, ECM regulating proteases can contribute to the angiogenic state during tumorigenesis by regulating growth factor release from the ECM.

To asses the capacity of FGFR1-FKBPv activation in invasion, the presence of myoepithelial cells, ECM, ECM proteases and vasculature were analyzed. The presence of the myoepithelial cell barrier in mammary glands from AP20187-treated mice was analyzed by immunofluorescence using the specific myoepithelial cell markers keratin-14 and smooth mucsle alpha-actin. In untreated wild-type mice a K14 and alpha-actin positive cell layer was observed between the luninal epithelium and stromal cell compartments. However, K14 positive myoepithelial cells surrounding the phenotypic lateral buds were not observed after two weeks of AP20187 treatment. Primary ducts associated with the lateral buds showed noncontiguous and patchy K14 staining along the duct. The loss of myoepthelial cells correlated with a reduction of proteinaceous ECM surrounding the FGR1-FKBPv induced lateral buds, as observed by Masson's trichrome staining. However, collagen IV (data not shown) and laminin (Fig3...) staining was present in the basal lamina surrounding the lateral buds. These data suggest that FGFR1-FKBPv activation can initiate the reorganization and breakdown of the ECM surrounding lateral buds.

ECM proteinases have been linked to angiogenesis through their regulation of growth factor availability to cell surface receptors. The ECM can function as a reservoir for endothelial growth factors such as VEGF and FGFs. In addition to assisting vascultature invasion, degradation of ECM proteins may increase the availability of growth factors to surrounding endothelial cells, thus initiating an angiogenic response. The matrix metalloproteinases (MMP) are a family of related Zn++ interacting proteases that have been increasingly implicated in tumorigenesis. *In vitro* analysis of MMP activity in FGFR1-FKBPv expressing mammary epithelial cells was used to determine if

MMPs may be regulated through FGFR1-FKBPv signaling. Using gelatin zymography, medium from serum starved FGFR1-FKBPv expressing HC11 mammary epithelial cells treated with AP20187 showed increased MMP activity over diluent treated cells. Gelatinase activity was inhibited when 5mM EDTA was added to the incubation buffer and conversion of proMMP to its cleaved form was observed when extracts were pre-treated with APMA. These data suggest that FGFR1-FKBPv activation in mammary epithelial cells can induce MMP-2 and MMP-9 activity.

Proliferation of the epithelium in the mammary gland requires concomitant changes not only in the underlying stroma but also in the vasculature network feeding the new growth. The relationship of the vasculature network with FGFR1-FKBPv induced lateral buds was observed *in situ* using confocal microscopy and FITC-lectin visualization of blood vessels. Mammary glands from transgenic mice treated for two weeks with AP20187 and injected with FITC-lectin into the left ventricle were frozen sectioned and stained with texas-red phalloidin. FITC-lectin staining within the extensive mammary gland vasculature network in addition to phalloidin stained ducts and lateral buds were clearly visible. Confocal imaging and software-rendered 3-dimensional reconstitution demonstrated a highly branched network of blood vessels surrounding lateral buds. Lateral buds were primarily associated with small tortuous vessels branching off of ductal vasculature. These data suggest sprouting angiogenesis from the existing ductal blood vessel network may be initiated through FGFR1-FKBPv signaling.

Discussion

During post-natal mammary gland development, branching morphogenesis occurs between 3-8 weeks of age, a time when FGF ligands and their receptors are expressed at highest levels. Additionally, the capacity of FGF ligands to induce mammary gland tumorigenesis has been observed in several mouse models. In these models, ligands are overexpressed under the control of unregulated mammary promoters, thus, the progressive steps initiating branching morphogenesis and transformation is difficult to study. Since FGFRs can be expressed on epithelial and stroma cells, the contribution between epithelial and stromal compartments in initiating these processes is difficult to elucidate. The targeting of a dimerizable FGFR to the mammary gland epithelium allows for analysis of direct effects elicited on the epithelium and indirect effects mediated through stroma. Furthermore, the inducibility of FGFR1 activation may limit the potential for cell adaptation to transgene signaling; a limitation that may occur in unregulated transgenic mouse models.

We have developed an inducible system to study FGFR activation and characterized its use *in vitro* and *in vivo*. In cultured cells, induced dimerization of FGFR resulted in the phoshorylation of signaling factors, inhibition of apoptosis and initiation of a proliferative response in mammary epithelial cells. These observations are consistent with previously published mechanisms for FGF signaling. To establish a mouse model for FGF signaling in the mammary gland we created transgenic mice that express the inducible construct under the control of the MMTV promoter. Using this transgenic mouse model we demonstrate for the first time that FGFR kinase activation in the

mammary epithelium is sufficient to induce hyperplastic and invasive lesions in the mammary gland.

The rapid four week timeline from the appearance of initial type-I to the invasive type-III lesions suggests that FGFR signaling in mammary epithelium has potent proliferative and anti-apopotic effects. In survival assays, activation of FGFR1 kinase inhibits caspsase-3 and activates the anti-apoptotic factor AKT. The conversion of single layer ductal epithtelium to multi-cell layers found in type-II and type-III lesions suggest that FGFR kinase activity *in vivo* can inhibit apoptosis associated with detachment of mammary epithelium from their basement membrane. In addition to the AP20187 induced anti-apoptotic response, a concomitant proliferative response was observed. In HC11 cells, AP20187 treatment induced a 2.5 fold increase in proliferation while a 10-12 fold increase was observed in transgenic mice. In AP20187 treated FGFR1-FKBPv mice no changes in apoptosis were observed by TUNEL staining when compared with untreated mice (data not shown), These data suggest that FGFR signaling can directly activate proliferation and survival signals within mammary epithelium to induce hyperplastic lesions.

By four weeks of AP20187 treatment invasive lesions appear in the mammary glands of transgenic mice. Several factors including MMP regulation, ECM remodeling and loss of myoepithelial cells may play a role in the invasive characteristics. Myoepithelial cells contribute to the production and maintenance of the ECM barrier that surrounds ductal epithelium, and secrete anti-angiogenic factors. Loss of myoepithilium and ECM is associated with invasive characteristics in breast cancer. The ECM not only provides a physical barrier between the mammary epithelium and stroma but also may

function in the regulation of angiogenesis. Hanahan has demonstrated that neutrophils expressing MMP-9 induce angiogenesis by releasing VEGF from the ECM, thus increasing its availability to endothelial cells. Interestingly, we have observed that mammary epithelial cells treated in culture with AP20187 increase secretion of MMP-9 and MMP-2. Consistent with these data, reduced ECM and increased vascular branching surrounding type-II/III lesions was observed in AP20187 treated transgenic mice.

Lateral branching in the mammary gland may function through a multi-step process of initiation and elongation requiring the synergistic action of several growth factor signaling pathways. Growth factors implicated in lateral budding include TGF-beta while ductal elongation may require IGF-1. The results of FGFR signaling described here are consistent with the a possible role for this growth factor family in initiating branching in the mammary gland. In this study, FGFR1 kinase activation elicited direct effects on mammary epithelium including regulation of proliferation, apoptosis, and MMP secretion. Increased MMP activity may function in remodeling of ECM, resulting in increased vascularization and invasive characteristics. Factors initiating branching morphogenesis and lateral budding would be required to educe similar effects on mammary epithelium during mammary gland development. It is possible that FGF signaling during ductal morphogenesis may contribute to proliferation, survival and invasion of mammary epithelium. Further studies using knock-out or dominant negative receptors will be required to characterize FGF function during ductal morphogenesis.

Dysregulation of FGF signaling pathways are associated with breast cancer. Overexpression of FGFRs has been observed in about 20% of breast cancer samples. Thus, the data presented here suggesting that direct effects of FGFR action on mammary

epithelium is sufficient to induce invasive-hyperplastic lesions has implications in the etiology of breast cancer. Further analysis of gene regulation by FGFR activation *in vivo* may additionally contribute to novel mechanisms of FGFR regulation in mammary gland transformation.

Methods

Plasmids and Cell Culture

The FGFR1 intracellular kinase domain was cloned by PCR amplification of a FGFR1 cDNA plasmid using the following primers: ..... The FGFR1 kinase domain was sequenced and sublconed into FKPBv plasmid. The FGFR1-FKBPv coding sequence was sublconed into p----- for retroviral transduction experiments and MMTV-KCR for use in the generation of transgenic mice. NIH3T3 cells were grown in growth media containing DMEM, 10% bovine calf serum and gentamicin. HC11 cells were grown in growth media containing RPMI supplemented with EGF, insulin, and genatmicin. Serum starvation media for NIH3T3 and HC11 cells contained only DMEM or RPMI, respectively, supplememtnted with either 30 nM AP20187 in ethanol or equal volume of ethanol diluent alone as control. 293T cell growth media contained DMEM, 10% fetal bovine serum and gentamicin. For transduction of cells, pMP-FGFR1-FKBPv and pMP-FKBPv plasmids were co-transfected with pCLeco into 293T cells using FuGene. Retroviral containing media was syringe filtered using a 40um filter unit, supplemented with .....polybrene and 5mls were added to 100mm plates of HC11 or NIH3T3 target cells. The target cells with retroviral media were spun in a .....centifuge at 1800 rpm for

30 minutes with a one-third rotation of the plate every 10 minutes for equal distribution of media over the plate. Target cells were then placed in normal growth media.

Survival and Proliferation assay

For NIH3T3 cell survival assays, FGFR1-FKBPv construct was co-transfected with pBKneo into NIH3T3 cells using Fugene. Stable cells were selected by supplementing growth media with 300ug of active G418/ml for 3 weeks. Pooled NIH3T3 cells were placed in serum-free DMEM media for 72 hours with daily media changes for morphological analysis. Images were captured using.....Caspase-3 activity was measured using a fluorgenic assay described by ..... FGFR1-FKBPv and FKBPv transduced NIH3T3 cells were treated with serum-free for 24 hours. Cells were lysed, incubated with ----peptide for three hours and fluorescence was measured at ---nM. Cell proliferation was measured by propidium iodide staining of cells treated in serum-free media for 48 hours. Treated cells were fixed for 30 minutes on ice in 100% ethanol, incubated with Rnase for 30 minutes at 370C followed by the addition of ..... propidium iodide and the measurement of DNA content by FACS.

Westerns and Zymography

The following antibodies were used for Western blot analysis: ..... Protein extracts from cultured cells were isolated using triton-X-100 extraction buffer (150mM NaCl. 1% triton-X-100, aprotinin, benzeanamine, antipain, trypsin inhibitor, PMSF, NaVanadate, NaF) pipet triteration and incubation on ice for 45 minutes. Protein extracts were resolved by PAGE and transferred onto PVDF membranes. For Western blot analsysis, membranes were blocked with 10% NFDM in TBST for 1.5 hours prior to antibody incubations. Antibodies were incubated in block buffer for 1hr-overnight.

Membranes were washed for 1 hour after each antibody incubation. Chemiluminsecence was developed following manufactures protocol. MMP-9 and MMP-2 activity were measured by gelatin zymography as described by .....

Immunofluorescence and Histology

The following antibodies were used for immunofluorescence:..... For immunofluorescence, tissue was fixed in fresh 4% paraformaldehyde in PBS for 2 hours prior to paraffin embedding. Immunofluorescence of paraffin embedded sections was carried out by dissolution of paraffin in xylene and grade ethanol rehydration to PBS. Antigen retrieval was performed for the following antigens.....by microwaving slides in100mM NaCitrate pH6.0 for 20 minutes followed by 20 minutes cooling at RT. For polyclonal antibodies, slides were then washed in PBS and blocked in 3% BSA for 1hour. For mouse monoclonal primary antibodies, MOM was used following manufactures protocol. Primary antibodies were incubated overnight at RT in a humidity chamber in block solution. Slides were washed in PBS after each antibody incubation for 30 minutes with three changes of PBS. Secondary antibodies were incubated in block buffer for 1 hour. Sections were mounted in vectashield containing dapi and imaged using..... Whole mounts were prepared as described by Seagroves, 1997. Hematoxylin and eosin sections were prepared as described by ..... Masson's trichrome staining followed the manufactures protocol

FITC-lectin Perfusion

AP20187 treated transgenic mice were anesthetized with Avertin and then lectin-FITC (*Lycopersicon esculentum* lectin, FITC conjugated B-1171)was slowly injected into the left ventricle of the heart. After 5 minutes, 5 mls of 1% paraformaldehyde/0.5%

gluteraldehyde was perfused followed by 5 mls of PBS. Tissue was then dissected and frozen in OCT compound. For confocal microscopy, thick 50um sections of OCT embedded tissue were cut and post fixed in 4% PFA/PBS for 10 minutes at RT. Sections were then permeablized with tritonX-100 for 10 minutes at RT, washed in PBS for 5 minutes and stained for 5 hours with phalloidin-Alexa 594 (Molecular Probes, A-12381) at 1:500 dilution in PBS. Sections were washed for 20 minutes in PBS, mounted with vectashield and imaged using .....

Figure Legend

Figure 1. (A) Schematic drawing of FGF induced dimerization of FGFR and conditional dimerization of FGFR1-FKBPv fusion protein by AP20187. (B) FGFR1-FKBPv is phosphorylated in response to AP20187 treatment. Phosphorylated proteins were immunoprecipitated using anti-phosphorylated tyrosine antibodies and characterized by Western using anti-HA epitope antibodies. (C) Western of protein extracts isolated from serum starved NIH3T3 and HC11 cells treated with AP20187 at several timepoints after treatment (in minutes). Anti-phosphorylated MAPK and AKT antibodies show activation of these signaling factors in response to AP20187 treatment. (D) Survival assay using serum starved NIH3T3 cells. R1 cells were stably transfected with FGFR1-FKBPv while pBKneo cells were stably transfected with pBKneo neomycin selection cassette. R1+ and pBKneo+ cells were treated with 30nM AP20187 while R1- and pBKneo- cells were treated with ethanol diluent. R1+ cells remained viable when serum starved. (E) Caspase-3 fluormetric-peptide cleavage assay of serum starved NIH3T3 cells transduced with FGFR1-FKBPv or FKBPv alone treated with increasing concentration of AP20187. AP20187 treated FGFR1-FKBPv cells showed reduced caspase-3 activity when

compared with FKBPv control cells. Data points represents quadruplicate wells. (F) Fold difference in proliferation of AP20187 treated serum starved NIH3T3 and HC11 cells. Spotted bars represent FGFR1-FKBPv and solid black bars FKBPv control. Data was normalized to cells treated with diluent. Proliferating cells were determined by greater than 2N DNA content as measured by propidium iodide staining and FACS analysis. Only FGFR1-FKBPv transduced HC11 cells showed an increase in proliferation in response to AP20187. Data points represent at least three independent analyses.

Figure 2. Whole mount and histological analysis of MMTV-FGFR1-FKBPv transgenic and wid-type littermates injected i.p. with AP20187 or diluent. Whole mounts of mammary glands (MG) at 4x magnification (A, C, E, G, I) and 10x magnification (B, D, F, H, J). (A and B) Wild-type mouse MG treated with AP20187. (C and D) Transgenic mouse MG treated with diluent. (E and F) Transgenic mouse MG treated with AP20187. (G and H) Ovex wild-type mouse MG treated with AP20187. (I and J) Transgenic ovex mouse MG treated with AP20187. (K) H&E stain of transgenic mouse MG treated with diluent. (L) Transgenic mouse MG treated with AP20187. (M) Wild-type mouse MG stained with anti-HA antibody and texas-red secondary (red) and dapi stained nuclei (blue). (N) Transgenic mouse MG stained with anti-HA antibody and dapi showing transgene localization in lateral buds.

Figure 3.. Three histologically distinct lesions are observed in AP20187 treated transgenic mouse mammary glands. (A) Panel shows gross mammary gland morphology by whole mounts (10X magnification), cellular detail by H&E stain (20X and 40X) and cellular polarity by immunofluorescence (100X) with anti-ZO-1 (texas-red) and laminin (FITC). Type I lesions initially appear by ten days of treatment (A and B) and is

characterized by a single layer of polarized mammary epithelial cells with large distinct lumens. Type II lesions appear starting at two-weeks of AP20187 treatment and is distinguished by multi-cellular epithelial layers and small collapsed lumens. Type III lesions are multi-cellular, invasive, well vascularized and have lost cell polarity.

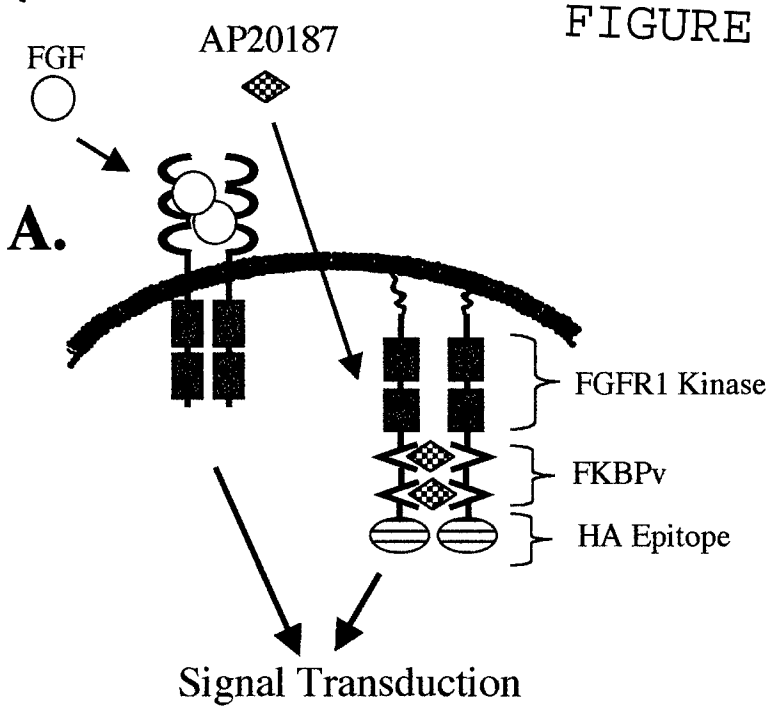
Figure 4. (A) Immunoprecipitation with anti-HA epitope antibodies and Western analysis using anti-phospho-tyrosine and HA-epitope antibodies of extracts from transgenic mice treated with AP20187 (R1+) or diluent (R1-) for two weeks. FGFR1-FKBPv (R1+) shows increased phosphorylation levels in AP20187 treated transgenic mice. (B) Western blot analysis showing increased phosphorylation levels of MAPK and AKT in AP20187 treated transgenic mice (R1+) over wild-type mice treated with diluent (wt). Positive control was FGFR1-FKBPv transduced NIH3T3 cells (R1+ NIH3T3) treated with AP20187. Immunofluorecesnce with anti-phosphorylated MAPK antibodies (texas-red) and dapi in wild-type (C) and transgenic (D) mice treated with AP20187 for two weeks. Anti-BrdU immunofluorescence from wild-type (E) and transgenic (F) mice treated with AP2087 for two weeks and pulsed with BrdU for two hours. Confocal microscopy and immunofluorescence of wild-type mid pregnant (G) and two-week AP20187 treated transgenic (H) mouse mammary glands using anti-E-cadherin (texas-red) and laminin (FITC) antibodies. Immunofluorescence shows loss of basolateral targeting of E-cadherin and multi-cellular layering in AP20187 treated transgenic mice.

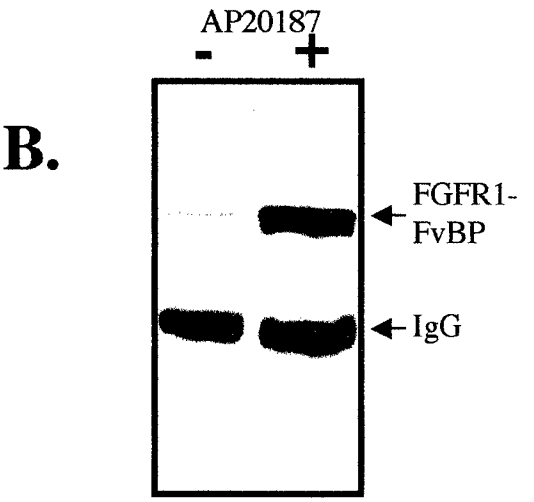
Figure 5. (A) FGFR1-FKPBv transduced HC11 cells were serum starved for 16 hours and treated with AP20187 or diluent in serum-free media for 24 hours. Media was collected, concentrated and loaded in equal volumes on a gelatin zymography gel. Media isolated from AP20187 treated cells had increased levels of MMP-9 and MMP-2 activity.

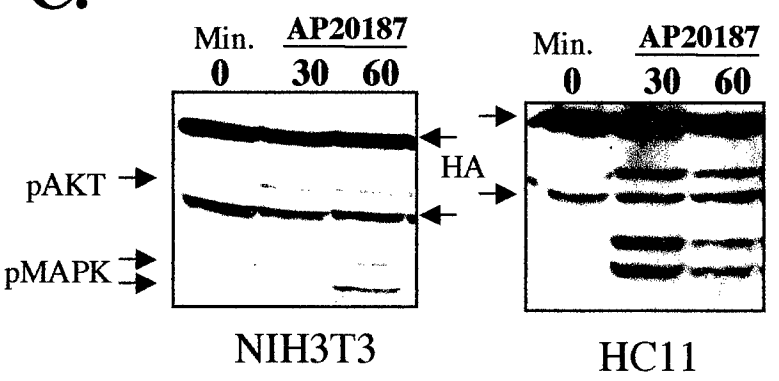
APMA treatment media increased the mobility of MMP-9 demonstrating conversion of proMMP-9 to active MMP-9. No APMA induced mobility shift was observed with MMP-2 suggesting only active MMP-2 was present. EDTA (5mM) treatment in the incubation buffer inhibited MMP activity. (B and C) Immunofluorescence using anti-keratin-14 antibodies (texas-red) staining demonstrate that AP20187 treated FGFR1-FKBPv mice have reduced myoepithelium surrounding lateral buds. (D) Masson's trichrome stain of untreated transgenic mouse mammary gland epithelium showing blue stained ECM (arrow) surrounding duct. (E) AP20187 treated mouse mammary gland with reduced ECM surrounding duct and lateral buds (arrows). (F and G) Confocal microscopy of vasculature network (FITC-lectin) surrounding mammary epithelium (texas-red-phalloidin) from AP20187 treated mice showing blood vessel branching (arrows) associated with lateral buds.

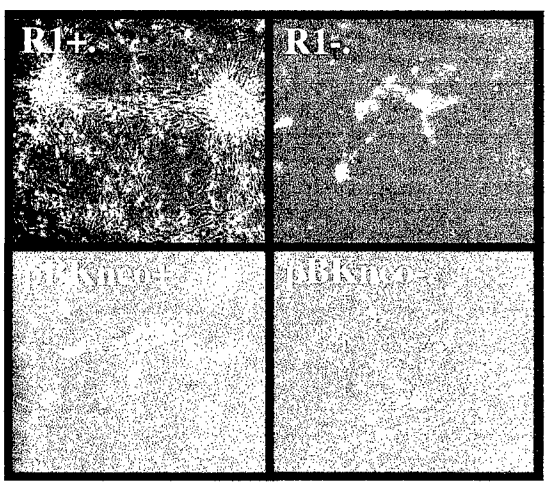
References

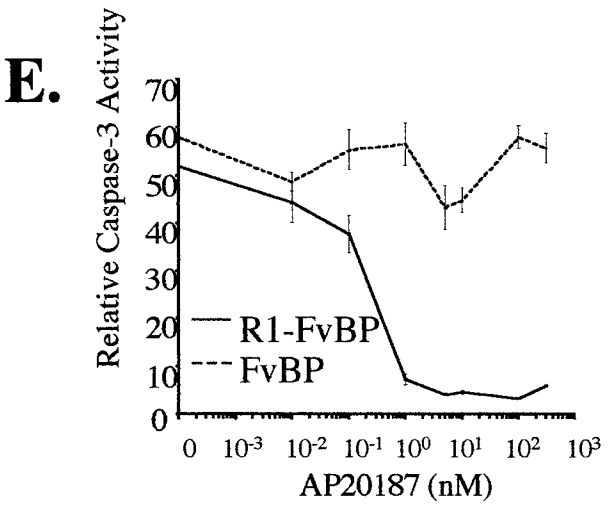
FIGURE 1


FGF
AP20187
A.
FGFR1 Kinase
FKBPv
HA Epitope
Signal Transduction


AP20187
-
+
B.
FGFR1-FvBP
IgG


C.
Min.
AP20187
0
30
60
pAKT
pMAPK
HA
NIH3T3
HC11


D.
R1+
R1-


E.
Relative Caspase-3 Activity
70
60
50
40
30
20
10
0
R1-FvBP
FvBP
0
10-3
10-2
10-1
100
101
102
103
AP20187 (nM)

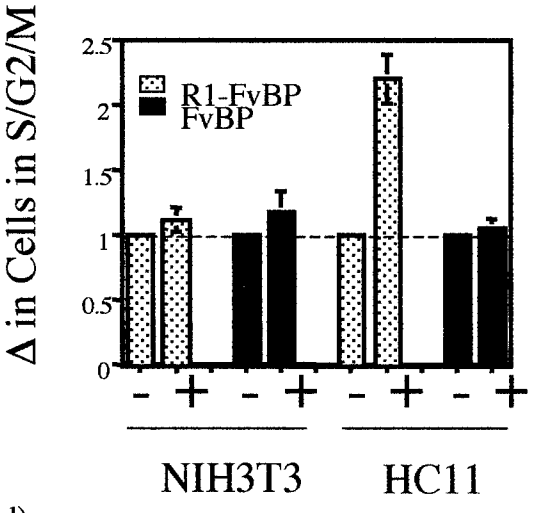

F.
Δ in Cells in S/G2/M
2.5
2
1.5
1
0.5
0
R1-FvBP
FvBP
- + - + - + - +
NIH3T3
HC11

FIGURE 2

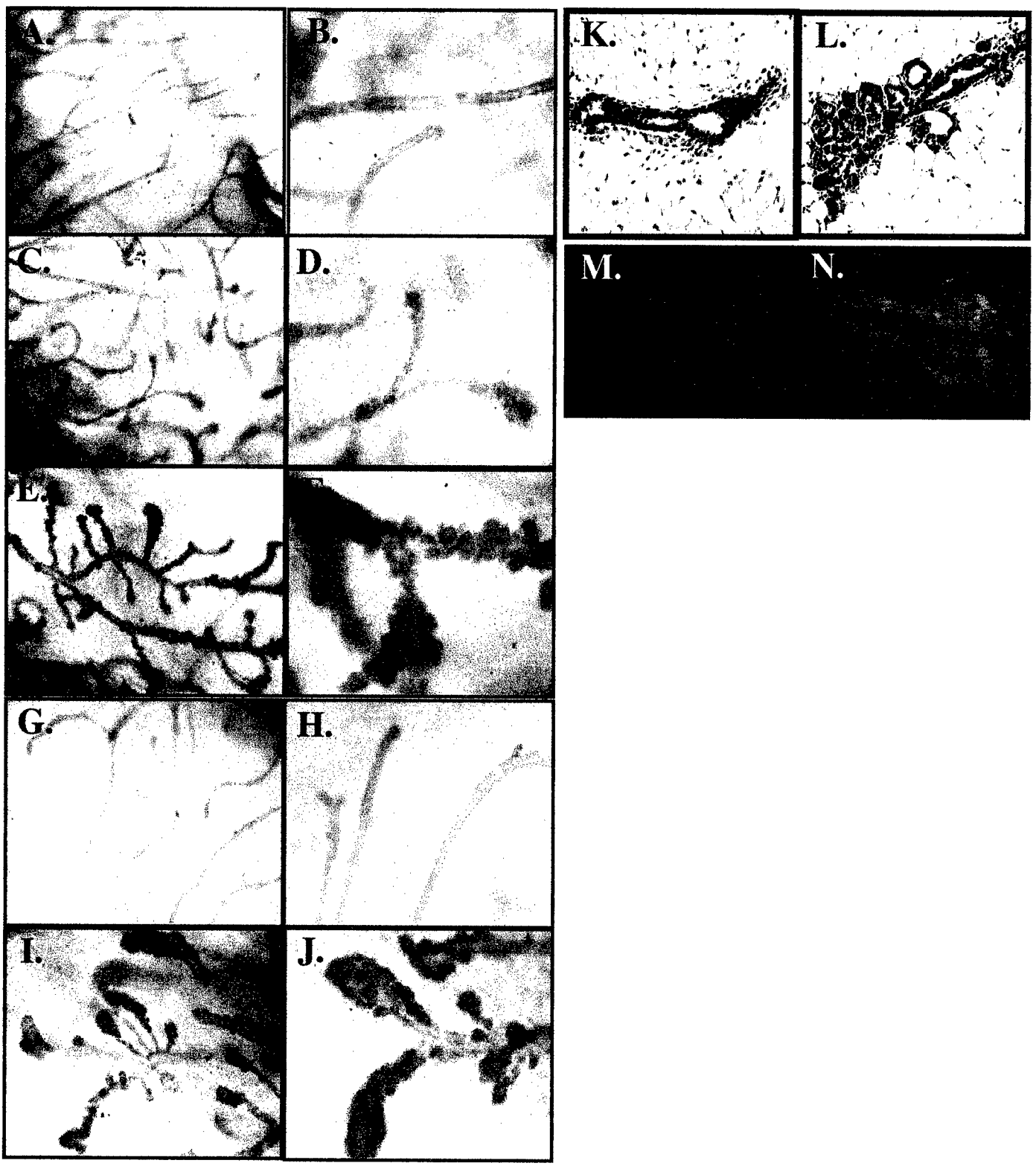
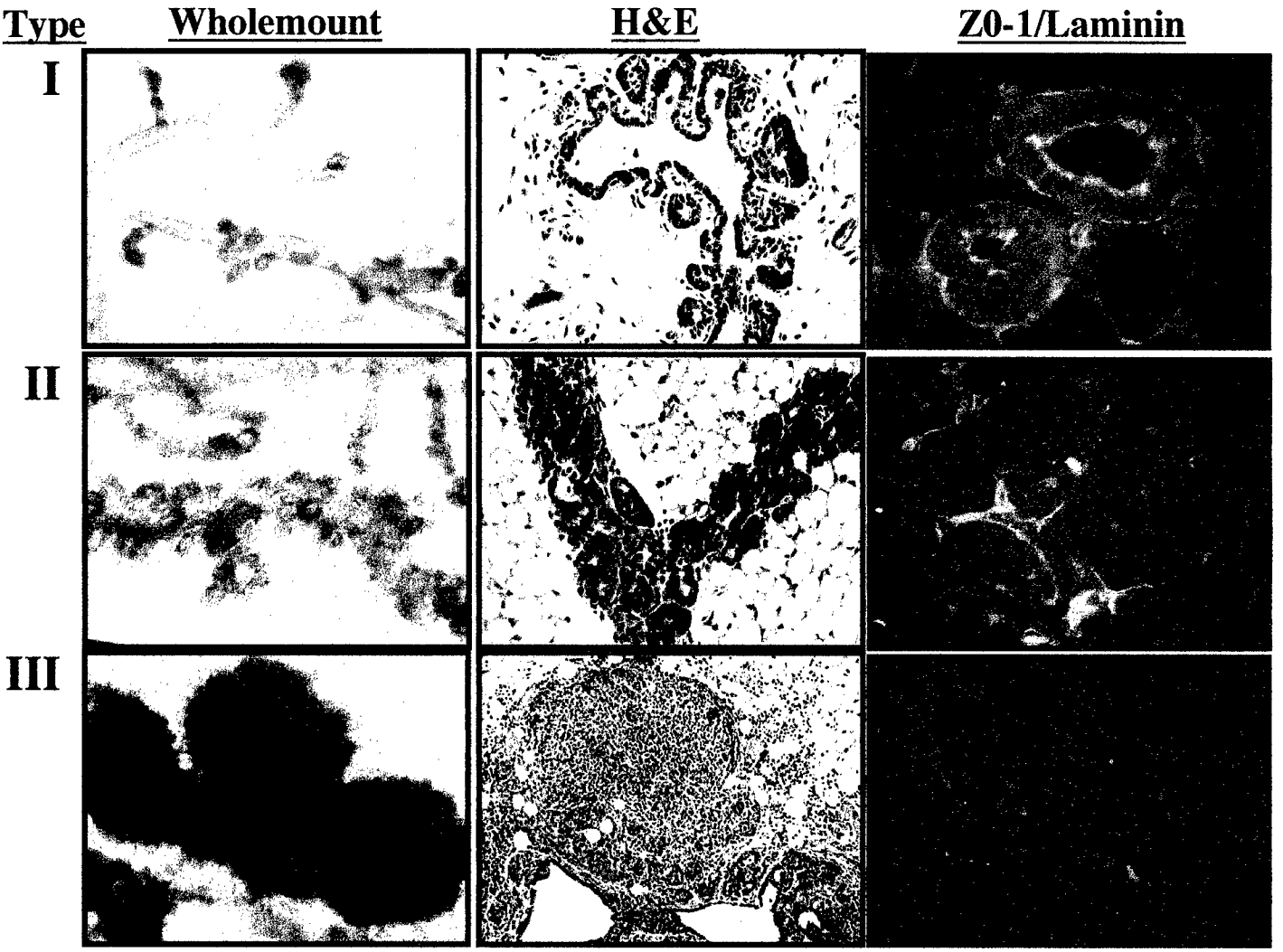


FIGURE 3

A.


Type
Wholemount
H&E
Z0-1/Laminin
I
II
III

B.

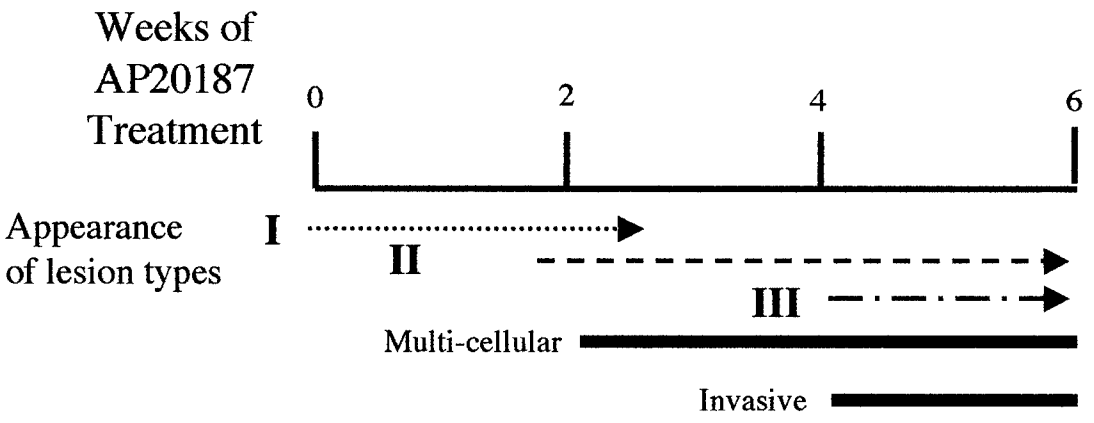

Weeks of AP20187 Treatment
0
2
4
6
Appearance of lesion types
I
II
III
Multi-cellular
Invasive

FIGURE 4

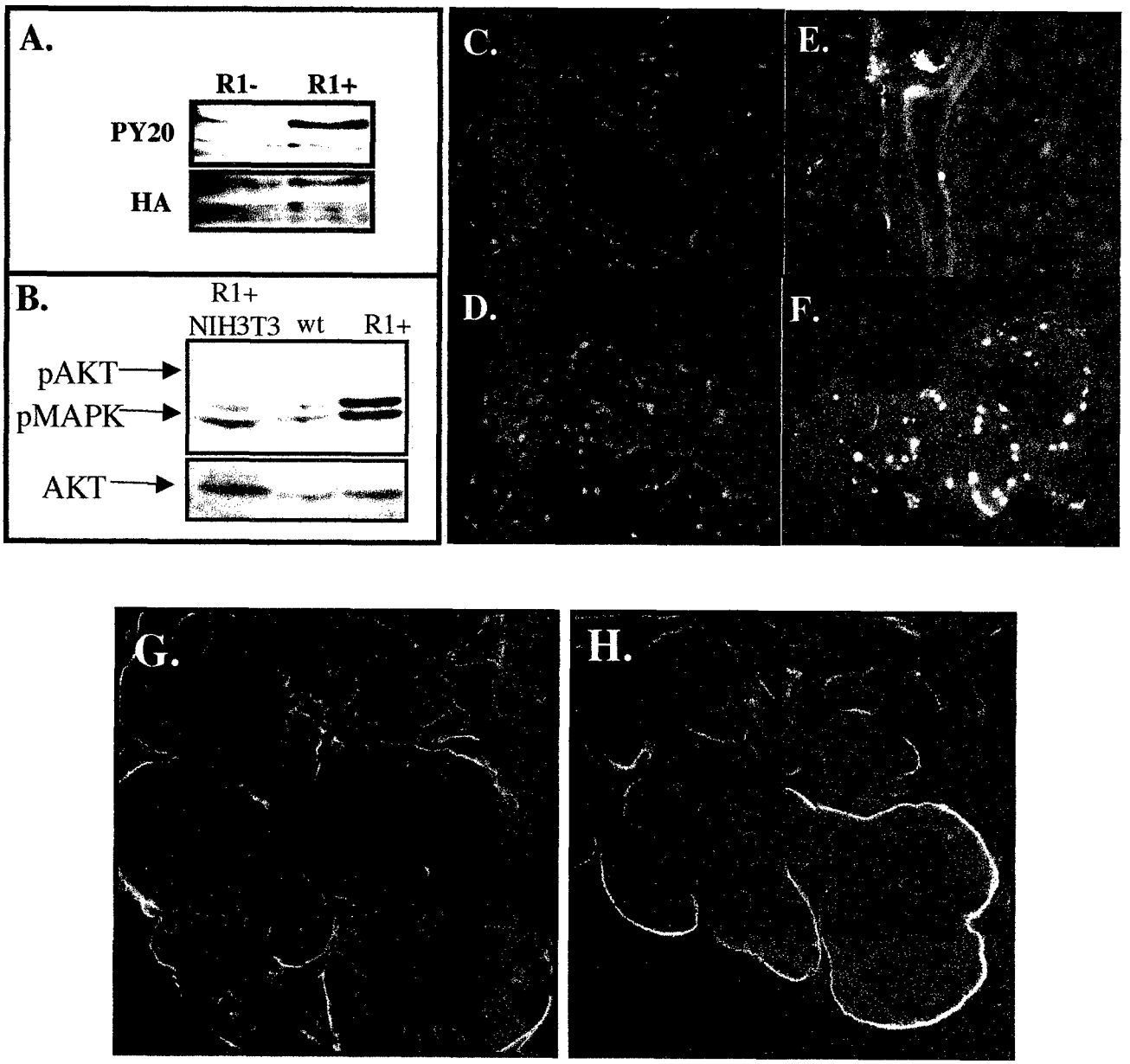
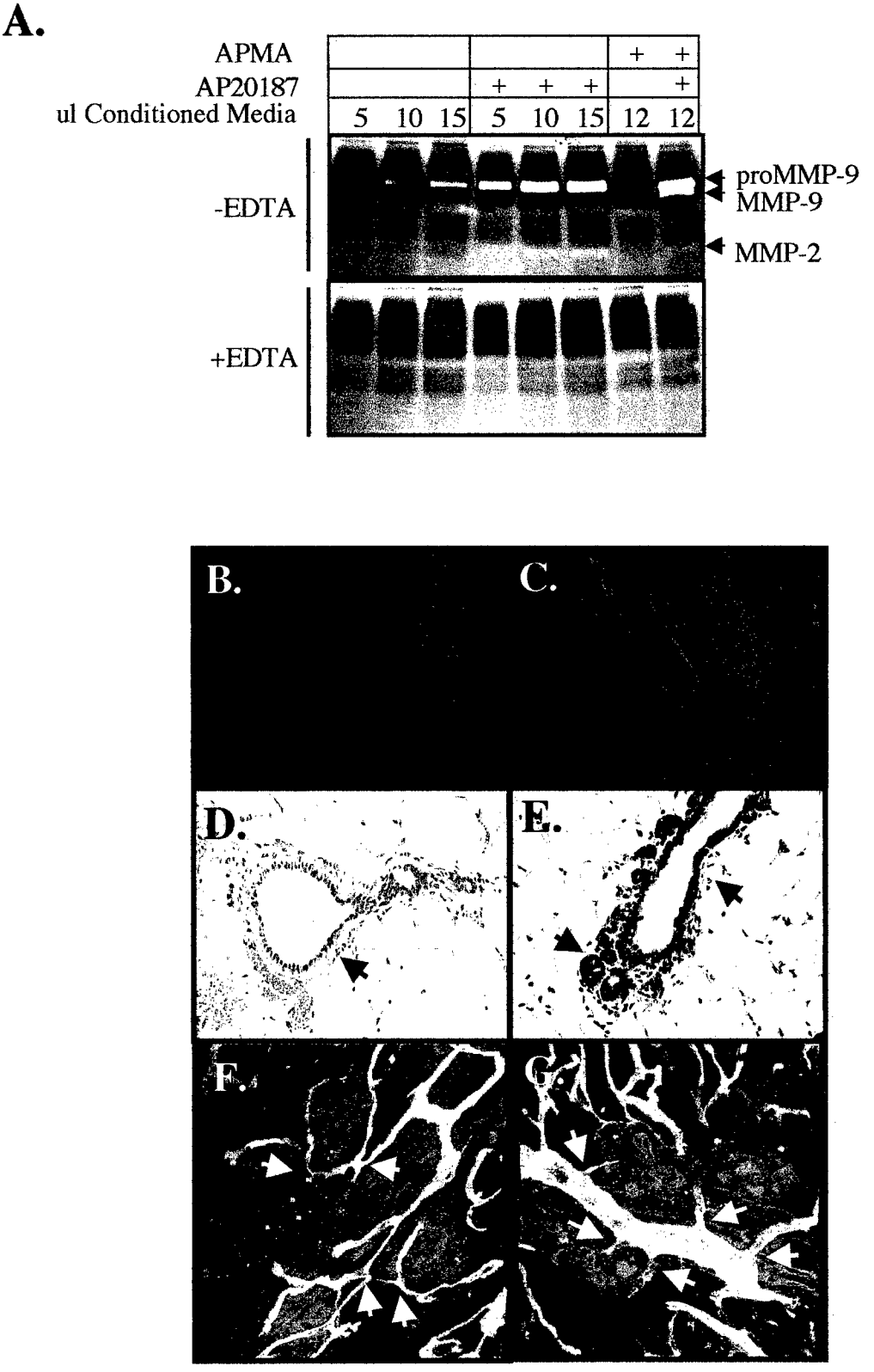


FIGURE 5


A.
APMA
AP20187
ul Conditioned Media
5 10 15 5 10 15 12 12
-EDTA
+EDTA
proMMP-9
MMP-9
MMP-2
B.
C.
D.
E.
F.
G.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLIS M. RINEHART
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